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In Vitro Susceptibilities of the AIDS-Associated Microsporidian *Encephalitozoon intestinalis* to Albendazole, Its Sulfoxide Metabolite, and 12 Additional Benzimidazole Derivatives

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Recent reports have described the successful treatment of *Encephalitozoon intestinalis* infection in AIDS patients with albendazole. However, this compound is rapidly metabolized in vivo to albendazole sulfoxide, and furthermore it is only 1 of about 15 commercially developed benzimidazole derivatives. To compare the activities of albendazole, albendazole sulfoxide, and other benzimidazoles, an in vitro system involving infection of green monkey kidney cell (E6) monolayers with *E. intestinalis* spores was developed. After 14 days, the effects of benzimidazoles on spore production were determined. Ten of fourteen derivatives tested, including albendazole, were inhibitory at concentrations of 1 to 10 ng/ml. Derivatives modified at the 1 or 2 position were less active. Albendazole sulfoxide was 1.7-fold more inhibitory than albendazole but significantly less toxic to E6 cells, a finding that explains the clinical efficacy of this compound. Potential alternatives to albendazole are discussed. No albendazole-resistant *E. intestinalis* mutants were obtained following in vitro selection.

Microsporidia are obligate intracellular parasites of a wide variety of vertebrate and invertebrate hosts. Infection typically begins with injection of the sporoplasm from a spore into the host cell via a polar tubule. Intracellular replication (merogony) is followed by the formation of environmentally resistant spores (sporogony), which are released upon lysis of the host cell. The taxonomic status of the phylum Microspora is currently unclear; while the highly divergent ribosome components (17, 28) and minimal complement of organelles imply that they are early-branching eukaryotes, recent studies of tubulin genes from microsporidia suggest a close relationship to fungi (19, 22).

Two microsporidial species have been repeatedly associated with intestinal infections in humans with AIDS: *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* (formerly *Septata intestinalis*) (2, 10, 26; for a review, see reference 29). It was reported in 1992 (7) that *Enterocytozoon bieneusi* infections were responsive to treatment with albendazole, 1 of 15 or so benzimidazole derivatives developed for use as anthelmintics in human and veterinary medicine and as fungicides in agriculture. Subsequent reports indicated that albendazole was only partially effective against *Enterocytozoon bieneusi* (2, 11) but highly effective against *E. intestinalis* and related *Encephalitozoon* species (1, 2, 9, 16, 23, 24, 30, 31). Benzimidazoles act by blocking the polymerization of tubulin into microtubules (for a review, see reference 20). While the selective toxicity of this group is based largely on differences in tubulin structure (18, 21), additional determinants of benzimidazole efficacy likely include extent of intestinal absorption, metabolism by the liver, and intracellular accumulation.

The recent development of in vitro culture systems for *E. intestinalis* (10, 26, 27) provides the opportunity to directly examine the inhibitory activity of benzimidazoles, along with their potential toxicity to host cells. We report here that albendazole, its primary metabolite albendazole sulfoxide, and eight other benzimidazole derivatives are inhibitory at low concentrations (1 to 10 ng/ml), while toxic concentrations range from 30 to >3,000 ng/ml.

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MATERIALS AND METHODS

Benzimidazoles. Parbendazole, oxibendazole, and albendazole sulfoxide were obtained from SmithKline Beecham (Philadelphia, Pa.), benomyl and carbendazim were from Du Pont (Wilmington, Del.), fenbendazole was from Hoechst-Roussel (Somerville, N.J.), oxfendazole was from Syntex (Palo Alto, Calif.), cambendazole was from Merck (Rahway, N.J.), flubendazole and cyclobendazole were from Janssen (Beerse, Belgium), and thiabendazole, albendazole, mebendazole, and nocodazole were from Sigma (St. Louis, Mo.). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C.

Cell culture and preparation of spores. *E. intestinalis* spores (strain CDC:V297, isolated from the urine of an AIDS patient [27]) and the E6 line of African green monkey kidney (Vero) cells were provided by G. S. Visvesvara (Centers for Disease Control and Prevention, Atlanta, Ga.). Cells were grown in 25-cm^2 culture flasks at 37°C in 5% CO_2 and typically subcultured every 96 h. The medium was minimum essential medium supplemented with L-glutamine (Gibco BRL, Bethesda, Md.), 5% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 2 μg of fungizone per ml, and 50 μg of gentamicin per ml. To prepare *E. intestinalis* spores, actively growing cells were infected at a ratio of three to four spores/cell, and the medium was collected and changed every 72 to 96 h for up to 4 weeks. The collected medium was centrifuged at 2,000 × *g* for 15 min, and the pellet was resuspended in fresh medium to a concentration of 20×10^4 spores/ml and stored at 4°C.

Assay of benzimidazole activity. Confluent monolayers of E6 cells were detached with trypsin-EDTA (Gibco BRL) and centrifuged at 1,000 × *g* for 10 min. The cell pellet was resuspended in fresh medium to a concentration of 5×10^4 cells/ml. One milliliter was placed in each well of a 24-well tissue culture plate, and the plates were incubated 12 to 15 h. Medium was removed and replaced with 1 ml of fresh medium containing *E. intestinalis* spores at a ratio of three to four spores/cell. One microliter of freshly diluted benzimidazole solution (1, 3, 10, 30, and 100 μg/ml in DMSO) was added; control wells received 1 μl of DMSO alone (0.1% final concentration). After 72 h, the medium was replaced with fresh medium and drug. This was repeated after additional incubations of 72 and 96 h; preliminary experiments indicated that essentially all spores that had failed to infect cells were removed by these three medium changes. Two weeks postinfection (following a final 96-h incubation), medium was collected and progeny spores were counted in a hemocytometer. Concentrations of drug inhibiting *E. intestinalis* replication to 50% of the level in control cultures (IC_{50}s) were estimated from plots of spore number versus log benzimidazole concentration.

Assay of benzimidazole toxicity. Uninfected E6 cultures (1 ml) were prepared in 24-well plates as described above. One microliter of benzimidazole solution in

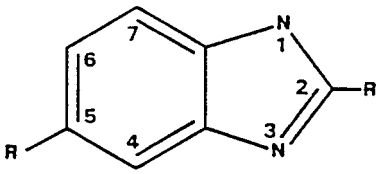

FIG. 1. General structure of benzimidazole compounds.

DMSO was added to a final concentration of 10 to 3,000 ng/ml, and incubation was continued for 72 h; controls received DMSO alone. Cultures were first examined microscopically for abnormal appearance and then detached with trypsin-EDTA and counted in a hemocytometer. IC_{50}s were estimated as described above.

RESULTS

Beauvais et al. (6), Ditrich et al. (12), and Franssen et al. (13) have described in vitro methods for testing drug activity against *Encephalitozoon cuniculi* or *Encephalitozoon hellem*. In their studies, infected cells were detected by staining and counted. We found it more convenient and accurate to count released spores rather than infected cells. The convenience derives from the ability to visualize spores without staining. Also, cultures can be sampled multiple times for spore production but can be stained only once. The improvement in accuracy derives from the absence of ambiguity in the counting of free spores (with a hemocytometer) in contrast to the counting of infected cells: infected cells contain variable numbers of progeny parasites, and variation among microscopic fields of cell monolayers can be large.

With two exceptions (thiabendazole and cambendazole), commercially developed benzimidazoles are 2-carbamate derivatives (Fig. 1; Table 1). The 5-position-unsubstituted benomyl and carbendazim are widely used as agricultural fungicides, while the 5-substituted derivatives include a number of anthelmintics important in human and veterinary medicine (e.g., mebendazole, albendazole, and fenbendazole). Nocodazole is unique in that it was originally developed as an anticancer agent due to its toxicity for dividing mammalian cells.

The inhibitory activities of benzimidazoles against *E. intestinalis*, expressed as IC_{50}s, are presented in Table 1. The most potent compound was nocodazole (IC_{50} = 1 ng/ml), but nine additional derivatives were also highly active, with IC_{50}s of $\leq$10 ng/ml. Albendazole was typical of this group, with an IC_{50} of 5 ng/ml. All of these are 2-position carbamate derivatives that differ widely in their 5 positions. Two compounds with 2-position thiazole, cambendazole and thiabendazole, were less active, as was benomyl, which is modified at the 1 position.

The E6 line of green monkey kidney cells was used as a host for *E. intestinalis*. To determine if observed benzimidazole activity was directed against the parasite or was an indirect effect of host cell toxicity, IC_{50}s were determined for uninfected E6 cells (Table 1). For all derivatives, the E6 cells were clearly less susceptible than *E. intestinalis*. However, the ratio of the IC_{50} for E6 to that for *E. intestinalis* varied from lows of 15 to 30 (parbendazole and nocodazole) to highs of >750 (oxfendazole and albendazole sulfoxide).

The use of benzimidazoles in the field as both fungicides and anthelmintics is seriously compromised by the emergence of resistant strains. The potential for development of benzimidazole resistance in *E. intestinalis* was examined by exposing E6 cultures infected with approximately 10^7 spores to gradually increasing concentrations of albendazole (3, 6, and 9 ng/ml) over a period of 3 months. After extended exposure to 6 ng of albendazole per ml, cultures had approximately twofold fewer spores than controls. The IC_{50} for these spores was determined to be 5 ng/ml, i.e., the same as that for unexposed spores (Table 1). After extended exposure to 9 ng of albendazole per ml, cultures appeared to be completely free of spores, and no viable *E. intestinalis* was recovered following a return to drug-free medium.

DISCUSSION

Several laboratories have examined the in vitro activity of albendazole against rabbit-derived *E. cuniculi* and determined its IC_{50} to be approximately 4 ng/ml (6, 12, 13). This is very close to the value presented here for *E. intestinalis* (5 ng/ml). Although the assays employed were different in several respects, it is likely that this agreement reflects the close taxonomic relationship between *E. cuniculi* and *E. intestinalis* revealed by rRNA analysis (3, 15) and, of particular relevance here, β-tubulin analysis (22). Franssen et al. (13) also reported results for two additional benzimidazole derivatives that were qualitatively similar to those obtained here; specifically, oxi-

TABLE 1. In vitro activities of benzimidazole derivatives against *E. intestinalis* and uninfected E6 host cells

Derivative	Side chain composition at position[a]:		IC_{50} (ng/ml)[b]		Ratio
	2	5	*E. intestinalis*	E6 host	
Nocodazole	$NHCOOCH_3$	CO-2-thienyl	1	30	30
Parbendazole	$NHCOOCH_3$	$CH_2CH_2CH_2CH_3$	2	30	15
Mebendazole	$NHCOOCH_3$	CO-phenyl	2	200	100
Albendazole sulfoxide	$NHCOOCH_3$	$SOCH_2CH_2CH_3$	3	>3,000	>1,000
Oxfendazole	$NHCOOCH_3$	SO-phenyl	4	>3,000	>750
Albendazole	$NHCOOCH_3$	$SCH_2CH_2CH_3$	5	300	60
Carbendazim	$NHCOOCH_3$	H	7	>3,000	>430
Fenbendazole	$NHCOOCH_3$	S-phenyl	10	200	20
Flubendazole	$NHCOOCH_3$	CO-4-fluorophenyl	10	NT[c]	
Oxibendazole	$NHCOOCH_3$	$OCH_2CH_2CH_3$	10	NT	
Thiabendazole	4-Thiazole	H	25	>3,000	>120
Cyclobendazole	$NHCOOCH_3$	CO-cyclopropyl	30	NT	
Cambendazole	4-Thiazole	$NHCOOCH(CH_3)_2$	75	NT	
Benomyl[d]	$NHCOOCH_3$	H	>100	>3,000	

[a] See Fig. 1.
[b] Data are averages of at least two independent experiments.
[c] NT, not tested.
[d] Benomyl includes a labile 1-position side chain [1-(butylamino)carbonyl].

bendazole and thiabendazole were highly and weakly active (IC_{50}s = 1.5 and 300 ng/ml), respectively.

Albendazole appears to be highly effective in treating *Encephalitozoon* infections, both intestinal and systemic, in AIDS patients (1, 2, 9, 16, 23, 24, 30, 31). This is at least partly due to the fact that albendazole, unlike most other benzimidazoles, is well absorbed following oral administration (for a review, see reference 25). However, following absorption albendazole is rapidly metabolized by the liver, primarily to albendazole sulfoxide. Thus, it is highly relevant that *E. intestinalis* is 1.7-fold more susceptible to this metabolite in vitro than to albendazole itself. This is not the case with several other opportunistic pathogens affecting AIDS patients. In vitro, both *Pneumocystis carinii* and *Cryptococcus neoformans* are highly susceptible to albendazole (4, 8) but only weakly susceptible to albendazole sulfoxide (unpublished data). Consequently, albendazole is weakly active or inactive against these organisms in mouse models (5, 14).

Mebendazole and thiabendazole are two additional benzimidazole derivatives approved for human use in many countries. Thiabendazole has relatively low activity against *Encephalitozoon* species (Table 1) (13) but is well absorbed. Conversely, mebendazole is highly active but poorly absorbed. Thus, it is unclear whether either would provide a suitable alternative to albendazole.

Like albendazole, fenbendazole is relatively well absorbed and rapidly metabolized, in this case to oxfendazole. Again, *C. neoformans* is highly susceptible to fenbendazole but not to oxfendazole (8), and fenbendazole was consequently inactive in a cryptococcosis mouse model (13a). In contrast, both compounds were highly active in vitro against *E. intestinalis*. Furthermore, of all the drugs tested, albendazole sulfoxide and oxfendazole were the least toxic to the monkey kidney cells. The highly active derivatives nocodazole and parbendazole, in comparison, were also highly toxic.

While fenbendazole would thus appear to be an acceptable alternative to albendazole, there are few if any studies on the use of this veterinary anthelmintic in humans. Similarly, while the fungicide carbendazim is highly active against *E. intestinalis* in vitro, there is little information on its pharmacologic properties. Albendazole therefore remains the best choice for treating *Encephalitozoon* infections. Unfortunately, the data presented here cannot be extrapolated with any confidence to the more common *Enterocytozoon bieneusi*, which appears to have much lower susceptibility to albendazole. It is clear, nevertheless, that benzimidazoles are diverse in terms of activity, toxicity, metabolism, and pharmacokinetics, and a derivative with improved anti-*Enterocytozoon bieneusi* activity could well exist.

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